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(57) Abstract

Host cell lipid molecules ("receptors") which permit binding of microorganisms are provided. Pharmaceutical compositions are formed from receptors in combination with a pharmaceutically acceptable carrier or diluent. The present invention also provides microbial proteins ("adhesins"), which mediate binding of microorganisms to host cells, and DNA sequences encoding adhesin proteins. Vaccines are formed from an adhesin protein in combination with a pharmaceutically acceptable carrier or diluent.

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Description

LIPID RECEPTORS FOR MICROORGANISMS AND CORRESPONDING ADHESINS, DNA SEQUENCES ENCODING ADHESINS, AND METHODS OF USE THEREOF

Technical Field

The present invention relates generally to receptors for microorganisms, complementary adhesins, adhesin genes, and methods of making and using these molecules.

Background of the Invention

The process by which microorganisms bind to host cells is called adherence or adhesion, and it is now well accepted that this mechanism is an important step in the initiation of microbial colonization and infection. Generally, the structures on the microorganism that mediate binding of infectious agents to host cells are called adhesins, and the host cell structures recognized by microbial adhesins are called receptors. Therefore, the presence of receptors on host tissue is just as much a determinant of microbial infectivity as is the microorganisms' display of adhesins. Attempts to identify high avidity adhesins and their corresponding host cell receptors, however, have been unsuccessful.

An example of a pathogenic microorganism of concern to humans is *Chlamydia trachomatis*. This microorganism is an obligate intracellular bacterial parasite of eucaryotic cells and is now known to be the most common sexually transmitted pathogen in industrialized societies (Moulder in Microbiology of Chlamydia, ed. A. L. Barron, pages 3-19, CRC Press, Boca Raton, Florida, 1988; Schachter in Microbiology of Chlamydia, pages 153-166, 1988). In the United States, it has been estimated that more than four million people contract chlamydial-related diseases each year (Eisner &

Monahan, Diagnostics and Clin. Testing 28:26-28, 1990). Salpingitis, ectopic pregnancy, infertility, chronic pelvic pain, premature labor, neonatal conjunctivitis, infant pneumonia, endemic trachoma, urethritis, and epididymitis have all been directly or indirectly related to infection by the organism (Schachter 1988; Eisner & Monahan, 1990).

Another example of a pathogenic microorganism of concern to humans is *Helicobacter pylori*. This microorganism is an infectious agent of the human stomach. Infection is associated with both primary, chronic-active gastritis, and peptic ulcer disease (Blaser, J. Infect. Dis. 161:621-623, 1990; Marshall, J. Infect. Dis. 153:650-657, 1986; Marshall et al., Lancet ii:1437-1442, 1988). Each year there are more than 300,000 new cases, 3,200,000 recurrences, and 3,200 deaths from duodenal disease in North America (Schefler, Statistics for Health Professionals, 1984). One and one half percent of all worker absenteeism in North America is a result of peptic ulcers (Jansen, Am. J. Med. 81:42-48, 1986). Antral gastritis is associated in turn with gastrointestinal carcinoma (Johansenn and Sikjay, Acta Path. Microbiol. Scan. 85:240, 1977) and recent studies have linked *H. pylori* with GI cancer (Parsonnet et al., 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Georgia, October 21-24, 1990, abst. no. 5).

Due to the difficulties in current approaches to the prevention and treatment of microbial diseases, there is a need in the art for improved methods and compositions for preventing and treating microbial diseases. The present invention fills this need, and further provides other related advantages.

Summary of the Invention

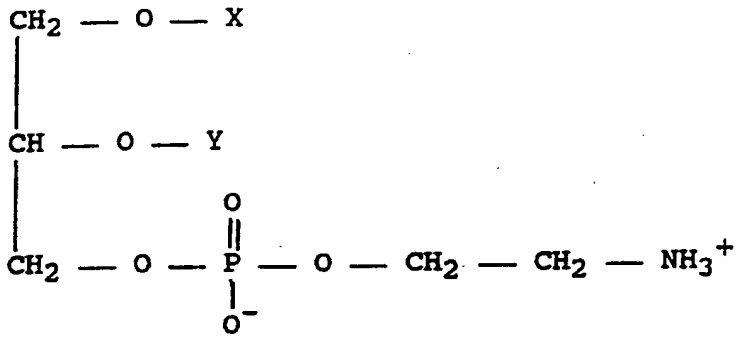
Briefly stated, the present invention provides a variety of compositions and methods related to lipid receptors for microorganisms, the complementary microbial

adhesins, and DNA sequences encoding adhesins. In one aspect, the present invention provides an isolated DNA molecule encoding an adhesin protein. Within one embodiment, the DNA molecule is cDNA. Within another embodiment, the DNA molecule is genomic DNA. Also disclosed is an isolated DNA molecule capable of specifically hybridizing with a DNA molecule encoding an adhesin protein.

In another aspect of the present invention, recombinant plasmids capable of expression in a host cell, the recombinant plasmid further comprising a DNA molecule encoding an adhesin protein, are provided. Suitable promoters and/or polyadenylation signals are also disclosed. In addition, eukaryotic and prokaryotic cells transfected with recombinant plasmids comprising a DNA molecule encoding an adhesin protein, and methods for producing an adhesin protein using host cells transfected or transformed with a suitable DNA molecule are also disclosed. A method for producing an adhesin protein comprises: introducing into a host cell a recombinant plasmid capable of directing the expression of an adhesin protein in the host cell; growing the host cell in an appropriate medium; and isolating the protein product, encoded by the DNA sequence, produced by the host cell.

Within a related aspect, the present invention discloses antibodies that specificaly bind to an adhesin protein. Preferred antibodies include monoclonal antibodies.

In another aspect of the present invention, an isolated receptor for microorganisms is provided. The receptor consists essentially of a plurality of phospholipids having the formula:



wherein X is $-\overset{O}{\overset{\|}{C}}-R$ or $-CH=CH-R'$;

Y is $-\overset{O}{\overset{\|}{C}}-R$; and

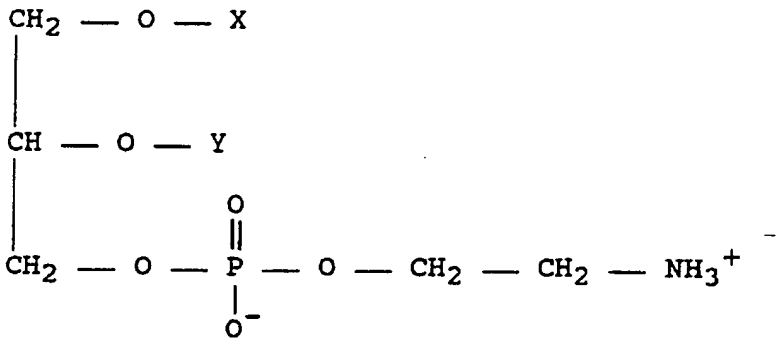
R′ is an alkyl group and R are alkyl, hydroxyalkyl or alkenyl groups of fatty acids. Also provided is a pharmaceutical composition comprising a phospholipid described above in combination with a pharmaceutically acceptable carrier or diluent.

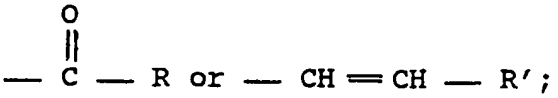
In yet another aspect of the present invention, methods for producing an isolated receptor for a microorganism are disclosed. In one embodiment, the method consists essentially of the steps of: extracting the lipids of HeLa cells to yield a lipid extract; contacting the lipid extract with DEAE-agarose exchange resin under conditions sufficient to permit binding; washing with methanol the exchange resin to which the lipid extract has been contacted; eluting a fraction with methanol containing 10-20 mM NH_4HCO_3; separating the fraction by silica gel preparative thin-layer chromatography; isolating a band positive for primuline and which binds a microorganism; washing the band with

chloroform; and eluting the receptor from the band with methanol.

Another aspect of the present invention provides methods useful for the screening for a microorganism which lacks an adhesin protein. In one embodiment, the method comprises the steps of: contacting a receptor described above with an aliquot of a selected microorganism under conditions and for a time sufficient to allow binding to occur; and detecting the presence or absence of a bound microorganism, thereby determining the presence or absence of an adhesin protein in the microorganism.

Within a related aspect, the present invention provides methods useful for the inhibition of microbial colonization. In one embodiment, the method comprises administering to a warm-blooded animal an effective amount of a composition comprising a pharmaceutically acceptable carrier or diluent in combination with a phospholipid having the formula:



wherein X is  ;

Y is  ; and

R′ is an alkyl group and R are alkyl, hydroxyalkyl or alkenyl groups of fatty acids.

In another embodiment, the method comprises administering to a warm-blooded animal an effective amount of the composition described above additionally including GalNAcβ1→4Galβ1→4Glc-Ceramide.

In another embodiment, the method comprises administering to a warm-blooded animal an effective amount of the composition described above additionally including Galβ1→3GalNAcβ1→4Galβ1→4Glc-Ceramide.

In another aspect of the present invention, microbial adhesin proteins are disclosed. Within one embodiment, a microbial adhesin protein is characterized by having been prepared by the process consisting essentially of: contacting a receptor described above with a microorganism preparation, wherein the preparation contains an adhesin protein, under conditions and for a time sufficient to allow binding between the receptor and the adhesin protein; and isolating the adhesin protein.

Within a related aspect, the present invention provides a vaccine comprising an adhesin protein described above in combination with a pharmaceutically acceptable carrier or diluent.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 shows the results of binding of *C. trachomatis* to lipids separated by thin-layer chromatography: (A) lipids visualized with orcinol reagent; (B) lipids visualized by overlaying with ^{125}I-labeled *C. trachomatis* followed by autoradiography for 48 h at -80°C; (C) lipids visualized by overlaying with ^{125}I-labeled *C. trachomatis* followed by autoradiography for 40 h at room temperature. Lane 1, 1 μg each of galactosylceramide (CMH), lactosylceramide (CDH),

trihexosylceramide (CTH), globoside (GL4), and gangliosides GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b; lane 2, 2 μg of phosphatidylethanolamine (PE) from HeLa 229 cells; lane 3, 2 μg of PE from bovine brain; lane 4, 2 μg of phosphatidylcholine; lane 5, 1 μg each of CDH, gangliotetraosylceramide (asialo GM1) and gangliotriaosylceramide (asialo GM2).

Figure 2 graphically illustrates the binding of *C. trachomatis* to immobilized lipids. ^{125}I-labeled *C. trachomatis* were incubated for 2 h at room temperature in microtiter wells coated with phosphatidylethanolamine extracted from HeLa 229 cells (-o-), gangliotriaosylceramide (-▪-), gangliotetraosylceramide (- □ -), phosphatidylcholine (- ⊡ -), lactosylceramide (- ◇ -), and globoside (-•-) at the indicated concentrations.

Figure 3 graphically illustrates the binding of *C. trachomatis* to immobilized phospholipids. ^{125}I-labeled *C. trachomatis* were incubated for 2 h at room temperature in microtiter wells coated with various phosphatidylethanolamines from HeLa 229 cells (-o-), soy bean (- ◇ -), yolk sac (-▪-), bovine brain (- ● -), bovine liver (- △ -), porcine liver (- ▲ -), and *E. coli* (- + -), phosphatidylcholine (- ⊡ -), and phosophatidylserine (- □ -) at the indicated concentrations.

Figure 4 shows the results of FAB mass spectrometry of: (A) *Chlamydia* receptor; (B) L-α-phosphatidylethanolamine from bovine brain ("bPE"); and (C) the receptor spectrum with the bPE spectrum subtracted. Samples were dissolved in triethanolamine and bombarded with xenon atoms with a kinetic energy of 8 keV and an accelerating voltage of 10 kV was used.

Figure 5 shows the results of ^{1}H-NMR spectroscopy of bPE and *Chlamydia* receptor. Spectra were obtained in CD_3OD at 27°C and chemical shifts are expressed relative to internal tetramethylsilane set to 0 ppm.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used herein.

Antibody - as used herein, includes an intact molecule, a fragment thereof, or a functional equivalent thereof; and may be genetically engineered. Examples of antibody fragments include $F(ab')_2$, Fab', Fab and Fv.

Complementary DNA or cDNA - a DNA molecule or sequence which has been enzymatically synthesized from the sequences present in an mRNA template, or a clone of such a molecule.

Plasmid or Vector - a DNA sequence containing genetic information which may provide for its replication when inserted into a host cell. A plasmid generally contains at least one gene sequence to be expressed in the host cell, as well as sequences which facilitate such gene expression, including promoters and transcription initiation sites. It may be a linear or closed circular molecule.

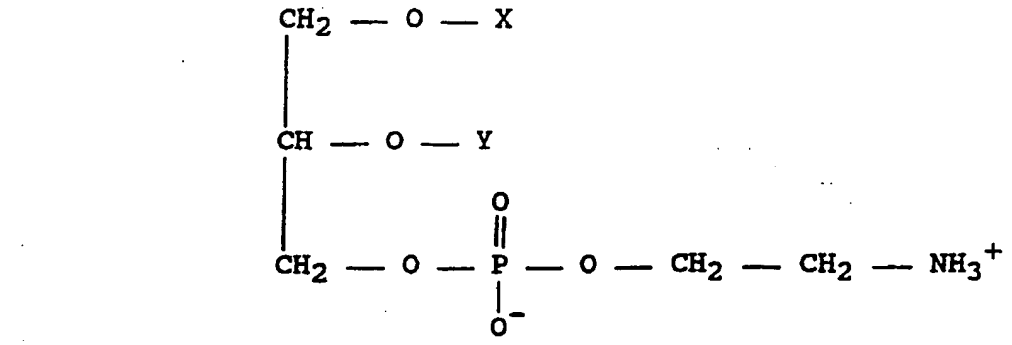
As noted above, an important step in the initiation of microbial colonization and infection is the adherence of microorganisms to host cells. Microorganisms bind specifically to host cell receptors. The microbial molecule which binds to host cell receptors, and thereby mediates the binding of the organism to the host cell, is called an "adhesin." Within the present invention, phospholipids are shown to be receptors for microorganisms and may be used to purify the corresponding adhesin proteins.

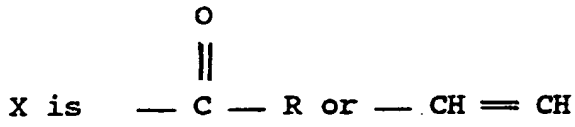
As disclosed within the present invention, a variety of microorganisms bind specifically to phospholipids isolated from human cells. Such microorganisms include *Streptococci*, *Borrelia*, *Haemophilus*, *Pseudomonas*, *Neisseria*, *Helicobacter*, *Pasteurella*, *Campylobacter*, *Erysipelothrix*, *Gardnerella*, *Propionibacterium*, *Treponema*, *Clostridium*, *Shigella*,

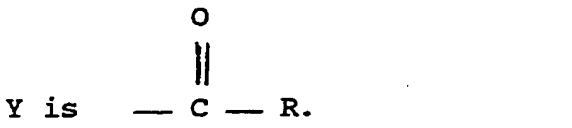
Bacteriodes, *Fusobacterium*, *Chlamydia*, *Mycobacterium*, *Yersina*, *Coxiella*, *Vibrio*, *Peptostreptococcus*, *Salmonella*, and *Mobiluncus*. Typically, the host receptors for these microorganisms may be found on epithelial cells of the respiratory tract, gastrointestinal tract, or reproductive tract, or on blood or epidermal cells. Representative cultured cell lines include human oropharyngeal epithelial cells, human tracheal epithelial cells, human endometrial cells, human embryonic amnion cells, human gingival fibroblasts, HeLa cells, and McCoy cells.

Purification of the phospholipids responsible for binding (i.e., "receptor") may be accomplished by a combination of extractions and chromatographic procedures. For example, briefly, cells (such as HeLa) are washed with phosphate buffered saline and extracted using chloroform/methanol/water. The extract is centrifuged, the pellet re-extracted, and the supernates combined ("lipid extract"). The lipid extract is applied to an anion exchange resin and, after a methanol wash, the receptor fraction is eluted with methanol containing 10-20 mM NH_4HCO_3. Following evaporation of the solvent and re-dissolving in 1:1 methanol-chloroform, the receptor fraction is further purified by preparative thin-layer chromatography, e.g., silica gel. The band containing the receptor is transferred to a glass column, washed with chloroform, and eluted with methanol. Purity may be assessed by analytical thin-layer chromatography.

The disclosure of the present invention shows that a purified receptor comprises the following phospholipid structure containing ethanolamine and several different fatty acids:







R represents alkyl, hydroxyalkyl and alkenyl chains of fatty acids, and R′ is an alkyl chain.

Fatty acids are typically abbreviated by numerical designations. For example, $CH_3(CH_2)_{12}CH_2CH_2COOH$ is 16:0 where the number to the left of the colon indicates the number of carbon atoms and the number to the right indicates the number of double bonds. The fatty acids identified in the purified receptor phospholipids from HeLa cells are 16:0, 18:1, 18:0, 20:4, and 18:9OH + 18:10OH, and are present in relative proportions of about 16%, 17%, 47%, 1%, and 19%, respectively. This collection of phospholipids binds microorganisms specifically and with high avidity. Phospholipids of this type, but missing one or more of these particular fatty acids, also bind microorganisms.

In addition to the phospholipids described above, microorganisms also bind strongly to specific glycolipids. In particular, the GalNAcβ1→4Galβ1→4Glc sequences found in the glycolipids asialo-GM1 and asialo-

GM2 appear to constitute a second receptor for microorganisms. Asialo-GM1 is the abbreviation for Galβ1→3GalNAcβ1→4Galβ1→4Glc-Ceramide and asialo-GM2 has the same structure minus the terminal Gal. Ceramides are sphingolipid bases which are acylated on the amine with a fatty acid.

The phospholipids of the present invention may be administered as a composition, which includes a pharmaceutically acceptable carrier or diluent, to a warm-blooded animal (such as a human) for inhibiting microbial colonization. Alternatively, such compositions may include one or more of the glycolipids described above. The precise optimal dose may vary, depending upon the particular phospholipid or glycolipid used. Generally, however, an effective amount will be from about 0.1 to about 10 mg per kg body weight. These phospholipids and glycolipids provide a means for preventing colonization by, for example, "fooling" a microorganism into binding to them (i.e., as an artificial receptor), rather than to a native receptor on a host cell.

Pharmaceutically acceptable carriers and diluents include water, physiological saline, liposomes, alcohols, dimethyl sulfoxide (DMSO) and mixtures thereof. A composition may be administered by a variety of routes, including oral, parenteral and transdermal administration. For oral administration, the composition may be in pill, capsule or liquid form. For administration by injection, physiological saline is a preferred diluent. For transdermal administration, DMSO is a preferred carrier.

The phospholipids of the present invention may also be used, individually or collectively, to screen for microorganisms which lack an adhesin protein. Briefly, one or more of the receptor phospholipids are incubated with a sample of a microorganism such that, if an adhesin protein is present in the microorganism, the microorganism will bind to the phospholipid. Suitable microorganism samples include intact cells and cellular fractions which

contain an adhesin, e.g., membrane preparations. The phospholipid may be attached to a solid support, such as a microtiter well or a chromatography resin (e.g., for column chromatography or thin-layer chromatography). It may be desirable to remove substantially any unbound microorganism, e.g., by washing the solid support with a suitable buffer.

Following binding of a microorganism sample to a receptor phospholipid, the presence of such binding may be detected in a variety of ways. For example, a microorganism may be detected directly where a reporter group is attached to the microorganism sample. Alternatively, a microorganism may be detected indirectly (e.g., where a reporter group is attached to a molecule, such as an antibody, which binds to a microorganism).

Detection of immunocomplexes formed between a microorganism and antibodies that specifically bind to it may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). The antibodies may be labeled (i.e., have a reporter group attached) or may be unlabeled. Unlabeled antibodies can be used in combination with labeled molecules that are reactive with immunocomplexes (e.g., protein A), or in combination with labeled antibodies (second antibodies) that are reactive with the antibody which binds to a microorganism. A number of immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,376,110; and 4,452,901.

Suitable reporter groups for direct or indirect detection include radioisotopes, fluorophores, enzymes, luminescers, and dye particles. These and other labels are well known in the art and are described, for example, in the following U.S. patents: 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402.

In addition to their screening and therapeutic uses, the receptor phospholipids described above may be used to isolate a microbial adhesin molecule. Briefly, a representative example of such an isolation begins with a microorganism preparation, such as a membrane extract, which contains an adhesin protein. Membrane extracts may be obtained using standard methodology. The extract is typically diluted 1:10 in a buffer containing bovine serum albumin. It may be desirable to additionally include a non-ionic detergent, such as octyl glucoside. The diluted extract is incubated with a receptor preparation, which has been immobilized onto a solid support, under conditions and for a time sufficient to allow binding to occur. Suitable solid supports include microtiter wells and chromatography resins, e.g., for column chromatography or thin-layer chromatography. The receptor may be immobilized onto a solid support by adsorption or covalent attachment. It will be evident to those skilled in the art that the receptor may be covalently attached in a variety of ways, including photoactivation and linker groups such as the homo- and hetero-functional reagents available from Pierce Chemical Co. (Rockford, Ill.).

The solid support, to which the adhesin molecules are bound via the receptor, is then washed to remove unbound material. The adhesin is then eluted with an elution buffer containing sodium dodecyl sulfate (SDS) or with a chaotropic agent, such as NaCl or KSCN. When a chaotropic agent is used, the eluate is dialyzed to remove the agent. Alternatively, an adhesin may be isolated by immunoaffinity using anti-adhesin antibodies. In this embodiment of a method for isolating an adhesin, antibodies directed against an adhesin are bound, preferably covalently, to a solid support such as a chromatography resin. The subsequent steps are similar to those described above, where a receptor is the affinity ligand.

The substantially pure adhesin may be analyzed by various analytical techniques, including SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Isolated adhesin proteins appear to be a family of related proteins which range in molecular weight (as determined by SDS-PAGE) from about 40,000 to about 65,000, depending upon the particular microorganism. For example, although an adhesin protein isolated from the outer membrane of *Haemophilus influenza* has a molecular weight of about 47,000, a higher molecular weight adhesin protein appears present in *Chlamydia* and *Helicobacter* and a lower molecular weight adhesin protein appears present in *Shigella flexneri* and *Streptomonas pneumonia*. Since the microorganisms expressing related adhesin proteins are evolutionarily widely divergent, the common expression of these proteins is likely due to convergent adaptation.

Polyclonal or monoclonal antibodies (MAbs) which are capable of specifically binding (*i.e.*, with a binding affinity of about 10^6 liters per mole) an adhesin protein may be produced. Briefly, polyclonal antibodies may be produced by immunization of an animal with an adhesin protein and subsequent collection of its sera. Immunization is accomplished, for example, by systemic administration, such as by subcutaneous, intraplenic or intramuscular injection, into a rabbit, rat or mouse. It is generally preferred to follow the initial immunization with one or more booster immunizations prior to sera collection. Such methodology is well known and described in a number of references. MAbs may be generally produced by the method of Kohler and Milstein (Nature 256:495-497, 1975; Eur. J. Immunol. 6:511-519, 1976). Briefly, cells of lymph nodes and/or spleens of an animal immunized with an adhesin protein are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin specific for the adhesin protein, and, like the myeloma cells, has the potential for indefinite cell division.

Suitable MAbs include those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (*i.e.*, antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies may be produced using methods well known by those skilled in the art. An alternative to the production of MAbs via hybridomas is the creation of MAb expression libraries using bacteriophage and bacteria (*e.g.*, Sastry et al., Proc. Natl. Acad. Sci. USA 86:5728-5732, 1989; Huse et al., Science 246:1275-1281, 1989).

An adhesin protein of the present invention may be used in a vaccine to prevent diseases associated with microbial infections. Interference with the interaction between receptor and adhesin is an effective basis for the prevention of the attachment of microorganisms to the receptors on host cells. Administration of an adhesin protein as a vaccine leads to an immune response in which antibodies which bind to the adhesin are produced. These antibodies inhibit binding of microorganisms to host cell receptors.

In addition to the adhesin protein (which functions as an antigen), it may be desirable to include other components in the vaccine, such as a vehicle for antigen delivery and immunostimulatory substances designed to enhance the protein's immunogenicity. Examples of vehicles for antigen delivery include aluminum salts, water-in-oil emulsions, biodegradable oil vehicles, oil-in-water emulsions, biodegradable microcapsules, and liposomes. Examples of immunostimulatory substances include N-acetylmuramyl-L-alanine-D-isoglutamine (MDP), lipopoly-saccharides (LPS), and glucan. It will be evident to those skilled in this art that an adhesin protein may be prepared synthetically and that a portion of the protein (naturally-derived or synthetic) may be used. When a peptide of the protein is used, it may be

desirable to couple the peptide hapten to a carrier substance, such as keyhole limpet hemocyanin.

The present invention also discloses isolated DNA molecules, including genomic DNA and cDNA, encoding an adhesin protein. The DNA of the present invention may be cloned into a microbial vector, such as a plasmid, or into a viral vector that may be harbored by a bacteriophage.

Preferably, DNA encoding an adhesin protein is identified by screening an appropriate genomic library (that contains microbial DNA) with a receptor or a monoclonal antibody to an adhesin. Such a library comprises colonies of a single type of microorganism, generally bacteria like *E. coli* K12 (HB101), into which pieces of foreign DNA have been inserted, generally by being incorporated into a plasmid, cosmid, or phage vector compatible with the microorganism. More specifically, the library comprises clones of vectors into which different sequences of DNA have been operably and recoverably inserted, each of the vectors containing only one sequence of DNA. The vectors may be plasmids, cosmids, or phage genomes. If necessary because of the type of library being used, segments of DNA will have been inserted into vectors in a manner that they will be expressed under appropriate conditions (i.e., in proper orientation and correct reading frame and with appropriate expression sequences, including an RNA polymerase binding sequence and a ribosomal binding sequence.) Suitable microorganisms are those that do not express the adhesin protein, such as *E. coli* HB101.

Once the library has been constructed, clones from the library are screened by contacting the clones with adhesin protein receptors or anti-adhesin antibodies under conditions and for a time sufficient to permit binding to the clones. Those clones which specifically bind the receptor or antibody are considered positive for DNA encoding an adhesin protein. The positive clones are isolated and the exogenous DNA sequence is recovered from

one of the clones. The sequence is evaluated to determine if it encodes an adhesin protein. For example, DNA may be sequenced according to the strategy of Sanger et al. (Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977), typically through the aid of an automated DNA sequencer (e.g., available from Applied Biosystems, Foster City, Calif.).

Preferably, the genomic library comprises bacteria, such as *E. coli* infected by phage. Preferably, the phage are bacteriophage lambda. Plaques produced by the phage-infected bateria are screened by adhesin protein receptors or monoclonal antibodies to identify those plaques containing bacteria that produce the adhesin protein. The screening involves contacting the plaques with the monoclonal antibody or the receptors to determine if binding has occurred, using standard techniques, e.g., by immunoassays. In this preferred embodiment, the positive clones are then isolated by purifying the positive plaques and inducing plasmid formation in the bacteria in the purified plaque with a helper phage according to standard techniques.

In an alternate preferred embodiment, colonies containing DNA that encodes an adhesin protein may be detected using DYNA Beads according to Olsvick et al. (29th ICAAC, Houston, Tex. 1989). The previously described receptors may be crosslinked to tosylated DYNA beads M280, and these receptor-containing beads are then used to adsorb to colonies expressing an adhesin protein. Colonies not expressing an adhesin are removed by washing, and this process is repeated to obtain an appropriate enrichment. Putative adhesin-expressing colonies are then plated and confirmed by metabolically labeling each colony with ^{35}S-methionine and testing the ability of the colony to bind to a receptor as previously described. The DNA from several adhering clones may be compared to identify shared sequences, and these shared sequences may be further subcloned and characterized.

In another alternate preferred embodiment, the gene for a specific adhesin may be localized and identified by constructing non-adherent mutants of a specific pathogen. This may be accomplished by creating mutants using a transposable element such as TnPhoA (as described in Manoil et al., Proc. Natl. Acad. Sci. USA 82:81129-8133, 1985). Alkaline phosphatase-positive mutants would indicate mutations within exported proteins. Since the adhesin for each microorganism is located on the outer membrane surface and therefore exported, this set of mutants contains a much reduced subset of mutants. The mutants are then screened for a loss in binding activity by procedures previously described.

It will be recognized by persons skilled in the art that a DNA sequence for an adhesin protein can be modified by known techniques in view of the teachings disclosed herein. For example, different codons can be substituted that code for the same amino acid as the original codon. Alternatively, the substitute codons may code for a different amino acid that will not affect the binding capability and/or immunogenicity of the protein, or which may improve its binding capability and/or immunogenicity. For example, oligonucleotide directed, site specific mutagenesis or other techniques to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis," Science 229:193-1210 (1985), can be employed. Since such modified DNA may be produced by the application of known techniques to the teachings contained herein, such DNA is within the scope of the present invention.

Moreover, it will be recognized by those skilled in the art that the DNA sequence (or fragments thereof) of the invention can be used to obtain other DNA sequences that hybridize with it under conditions of moderate to high stringency, using general techniques known in the

art. For example, stringent hybridization and washing condutions are described by Suggs et al. (in Developmental Biology Using Purified Genes, Eds. D. Brown and C.F. Fox, p. 683, Academic Press, N.Y., 1981). Accordingly, the DNA of the present invention includes such DNA.

The DNA of the present invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform a microorganism for the expression and production of an adhesin protein. For example, recombinant plasmids capable of integration into a host cell comprise a promoter followed downstream by a DNA sequence encoding an adhesin protein. It may be desirable to include a polyadenylation signal downstream from the DNA sequence. One embodiment of a method for producing an adhesin protein comprises introducing into a host cell a DNA sequence encoding an adhesin protein. The host cells are grown in an appropriate medium and the protein product encoded by the DNA sequence produced by the host cell is isolated. Examples of techniques known in the art include those disclosed in U.S. Patent Nos.: 4,440,859, issued April 3, 1984 to Rutter et al.; 4,530,901, issued July 23, 1985 to Weissman; 4,582,800, issued April 15, 1986 to Crowl; 4,677,063, issued June 30, 1987 to Mark et al.; 4,678,751, issued July 7, 1987 to Goeddel; 4,704,362, issued November 3, 1987 to Itakura et al.; 4,710,463, issued December 1, 1987 to Murray; 4,757,006, issued July 12, 1988 to Toole, Jr. et al.; 4,766,075, issued August 23, 1988 to Goeddel et al.; and 4,810, 648, issued March 7, 1989 to Stalker. It will be evident to those skilled in the art that it is not necessary to use the entire sequence when producing recombinant adhesin proteins. Further, DNA of the present invention may be joined to a wide veriety of other DNA sequences for introduction into an appropriate host cell. The companion DNA would depend upon the nature of the host cell, the manner of the

introduction of the DNA into the host cell, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognized by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Not all of the hosts may be transformed by the vector. Therefore, it may be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Transformed host cells of the present invention express adhesin proteins. Such cells are cultured by known techniques, and the proteins are recovered by known techniques. Depending upon the host and expression system used, the recombinant proteins of the present invention may be part of a fusion protein produced by the transformed host cells. Such proteins are recovered by known techniques, and the undesired part may be removed by known techniques. Alternatively, the fusion protein itself may be more immunogenic than the recombinant protein or polypeptide alone and, therefore, may itself be useful, e.g., in a vaccine.

If desirable, the adhesins can be further purified by the application of standard protein purification techniques, modified and applied in accordance with the teachings described herein. Such techniques include electrophoresis, centrifugation, gel

filtration, precipitation, dialysis, chromatography (including ion exchange chromatography, affinity chromatography, immunoadsorbent affinity chromatography, reverse-phase high performance liquid chromatography, and gel permeation high performance liquid chromatography), isoelectric focusing, and variations and combinations thereof.

One or more of these techniques are employed sequentialy in a procedure designed to separate molecules according to their physical or chemical characteristics. These characteristics include the hydrophobicity, charge, binding capability, and molecular weight of the protein. The various fractions of materials obtained after each technique are tested for their ability to react with the adhesin receptors. Those fractions showing such activity are then subjected to the next technique in the sequential procedure, and the new fractions are tested again. The process is repeated until only one fraction reactive with the receptors remains and that fraction produces only a single band when subjected to polyacrylamide gel electrophoresis. The preferred techniques include those identified and described in U.S. Patent No. 4,446,122, issued May 1, 1984 to Chu et al., which is incorporated herein by reference. Preferably, the adhesins are purified by receptor affinity chromatography or monoclonal antibody affinity chromatography.

Adhesins of the present invention may be modified by protein modification techniques. Suitable techniques are well known and include those described by Means and Feeney (Chemical Modification of Proteins, Holden-Gay, 1971). Such modifications include breaking the protein into fragments that contain at least one active site or the addition, substitution, or deletion of one or more amino acids to the protein or a fragment thereof. Preferably, such protein derivatives are immunologically cross-reactive with an adhesin protein, thus being capable of eliciting an antigenic response to a

microorganism in an animal host. Such modifications may enhance the immunogenicity of the protein or have no effect on such activity. Modification techniques additionally include those disclosed in U.S. Patent No. 4,526,716, issued July 2, 1985 to Stevens.

Adhesin proteins of the present invention may contain one or more amino acid sequences that are not necessary to its immunogenicity. For example, only the amino acid sequences of a particular epitope of the antigen may be necessary for immunogenic activity. Unwanted sequences can be removed by techniques well known in the art. For example, unwanted amino acid sequences can be removed via limited proteolytic digestion using enzymes such as trypsin, papain, or related proteolytic enzymes.

Alternatively, polypeptides corresponding to various immunogenic epitopes and/or the receptor binding site of the protein may be chemically synthesized by methods well known in the art, given the teachings contained herein. These include the methods disclosed in U.S. Patent No. 4,290,944, issued September 22, 1981 to Goldenberg.

Modified proteins may be prepared that are substantially homologous to an adhesin protein through the use of known techniques and routine experimentation in view of the teachings contained herein. Such a protein may be identified by the fact that it will bind to antibodies that were generated by use of an adhesin protein of the invention.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

BINDING OF CHLAMYDIAL ORGANISMS TO LIPIDS

A. Growth and Radiolabeling of a Chlamydial Organism

A cervical isolate of *C. trachomatis* serovar E was grown in HeLa 229 cells and chlamydial elementary bodies (EBs) were purified by a modified procedure of a renograffin gradient procedure (Caldwell et al., Infect. Immun. 31:1161-1176, 1981; Bavoil et al., Infect. Immun. 44:478-485, 1984). The purified EBs were washed twice with phosphate buffered saline (PBS) and the density of the EBs was adjusted to that of McFarland No. 3 tube with PBS.

Chlamydial organisms were radioiodinated as described for bacteria (Krivan et al., Arch. Biochem. Biophys. 260:493-496, 1988), with minor modifications. Briefly, 0.5 ml of the chlamydial suspension were reacted with 0.5 mCi of $Na^{125}I$ at an ice bath in a tube (10 x 75 mm) coated with 0.1 mg of Iodogen (Pierce Chemical Co., Rockford, Ill.). After 4 to 5 min, the suspension was transferred to a centrifuge tube containing 5 ml of Tris-BSA buffer (0.05 M Tris hydrochloride [pH 7.8] containing 0.15 M NaCl and 1% bovine serum albumin). The tube was centrifuged at 30,000 x g for 30 min at 4°C and the supernatant fluid was removed. The pellet was resuspended in 6 ml of Tris-BSA buffer and the centrifugation was repeated. The supernatant fluid was removed and the pellet was resuspended in 5 ml of Tris-BSA buffer. The radioactivity of the suspension was adjusted to 2×10^6 cpm/ml for a chromatogram overlay assay and to 4×10^6 for a solid-phase binding assay with RPMI-BSA (RPMI 1640 medium [GIBCO Laboratories, Grand Island, N.Y.] containing 1% BSA).

B. Sonic Extraction of Chlamydial Organisms and Radiolabeling of the Fraction

The purified EBs were sonicated for 7 min in an ice bath using a microtip followed by centrifugation at 4°C for 30 min at 30,000 x g. The supernatant fluid was designated as sonic extract and its protein concentration was determined by BCA protein assay (Pierce Chemical Co., Rockford, Ill.) using bovine serum albumin as a standard.

The sonic extract was radiolabeled as described for protein (Magnani et al., Meth. Enzymol. 83:235-241, 1982) with minor modifications. Briefly, 10 µg protein in 30 µl PBS of the sonic extract was mixed with 100 µl of 0.3 M sodium phosphate buffer in an Iodogen-coated tube as described above. One mCi of $Na^{125}I$ was added to the tube and the sonic extract was iodinated for 2 min in an ice bath with frequent shaking. The reaction mixture was transferred to a PD-10 Sephadex G-25M column (Pharmacia LKB, Upssala, Sweden) which was pre-equilibrated with Tris-BSA. After the mixture passed through the column, 1 ml of Tris-BSA was added to the top of the column and this was followed by adding more Tris-BSA. The first 1 ml was discarded and the next 5 ml was collected. The radioactivity of the sonic extract was adjusted to 2×10^6 cpm/ml for a chromatogram overlay assay and to 4×10^6 cpm/ml for a solid-phase binding assay with RPMI-BSA.

C. Chromatogram Overlay Assay for Binding of Chlamydial Organisms to Lipids

The overlay assay was performed as described for bacteria (Krivan et al., Arch. Biochem. Biophys. 260:493-496, 1988). Briefly, lipids were chromatographed on aluminum-backed silica gel high-performance thin-layer plates (HPTLC; E. Merck AG, Darmstadt, Federal Republic of Germany), and developed with chloroform-methanol-0.25% aqueous KCl (5:4:1). The plate was coated with polyisobutylmethacrylate (0.1% in hexane), air-dried, soaked for 1 h in Tris-BSA buffer, and overlayed for 2 h

at room temperature with ^{125}I-labeled either chlamydial organisms or chlamydial subcellular fraction as described above. The plates were gently washed to remove unbound organisms, dried, and exposed for 40 h to XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

D. Solid-Phase Assay for Binding of Chlamydial Organisms to Lipids

The solid-phase binding assay was performed as described by Krivan et al., Arch. Biochem. Biophys. 260:493-496, 1988. Briefly, serial dilutations of purified lipids in methanol (25 µl) containing cholesterol and phosphatidylcholine (0.1 µg each) were added to polyvinylchloride microdilution wells (Falcon 3919; Becton Dickinson and Co., Paramus, N.J.) and dried by evaporation. The wells were blocked with Tris-BSA for 1 h, rinsed with RPMI-BSA twice, and incubated with 25 µl of ^{125}I-labeled either chlamydial organisms or its subcellular fraction for 2 h at room temperature. After the wells were washed five times with PBS, the polyvinylchloride wells were cut with scissors and placed in counting tubes. Binding was quantified in a gamma counter.

Example 2

PURIFICATION AND CHARACTERIZATION OF *CHLAMYDIA* RECEPTOR

A. Purification

HeLa 229 were grown in TC-175 cm^2 flasks and harvested either by mild trypsinization or scrapping. Cells were washed three times in 0.0067 M phosphate buffered saline (PBS, pH 7.2). Total lipids of HeLa 229 cells were extracted by adding three volumes (to the wet weight of the cells) of deionized water, ten volumes of methanol and five volumes of chloroform. The mixture was ultrasonicated for 2 min and incubated overnight on a rocking bed at room temperature. The extract was

centrifuged at 4°C for 5 min at 2,000 rpm. Supernatant fluid was saved and the pellet was resuspended in the same volumes of deionized water, methanol and chloroform. The suspension was ultrasonicated for 30 min and supernatant fluid was collected by centrifugation. The first and second supernatant fluids were combined in a round bottom flask and dried on a rotary evaporator.

The dried total lipid extract of HeLa 229 cells was solubilized in 1:1 methanol-chloroform (0.5 ml per 1 gram wet weight of the cells). A portion of the total lipid was stored at -20°C for later analysis. The rest was dried under nitrogen and dissolved in the original volume of chloroform-methanol-water (30:60:8). The total lipids of HeLa 229 cells were applied to a DEAE-Sepharose CL-6B column (Pharmacia AB, Uppsala, Sweden) and allowed to bind for 20 min. Neutral lipids were eluted first with five gel volumes of methanol, then the fraction containing *Chlamydia* receptor ("receptor") was eluted with five gel volumes of methanol containing 10-20 mM NH_4HCO_3. Fractions containing receptor migrated between CMH and CDH as analyzed by HPTLC and were weakly orcinol positive. The phospholipid fractions were verified to contain the *Chlamydia* receptor by the ability to bind radiolabeled elementary bodies as analyzed by the chromatogram overlay assay described in Example 1. Other acidic lipids were eluted with five gel volumes of methanol containing 0.5 M NH_4HCO_3. Each lipid elution was dried on a rotary evaporator and redissolved in 1:1 methanol-chloroform.

The fraction containing receptor was further purified by chromatographing on Silica Gel G-2000 microns thin-layer preparative plates (Anal. Tech, Newark, N.J.), developed with 5:4:1 chloroform-methanol-aqueous 0.25% KCl. The plates were sprayed with primuline and examined by a longwave U.V. light. The band containing receptor stains positive with primuline and binds *Chlamydia* in the chromatogram overlay assay as described in Example 1. The receptor band was scrapped out, minced and packed in a

glass column. The column was washed with five gel volumes of chloroform, then receptor was eluted with ten gel volumes of methanol. This elution was rotary evaporated and redissolved to 1/2 of the original volume with 1:1 methanol-chloroform. The purity of receptor was examined by thin-layer chromatography, and its ability to function as the *Chlamydia* receptor was verified by chromatogram overlay assay, both procedures as described in Example 1.

B. Analyses

1. Chemical

Amino acid analysis (according to the procedures of Spackman et al., Anal. Chem. 30:1190-1206, 1958) of receptor revealed an absence of amino acids, but the presence of ethanolamine (10%-15% by weight). The results of fatty acid analysis (according to the procedure of Gaver & Sweeley, J. Am. Oil Chem. 42:294-298, 1965) of "bPE" (L-α-phosphatidylethanolamine from bovine brain, P8673 lot No. 69F-8365-1, Sigma Chemical Co., St. Louis, MO), and receptor are summarized in Table 1.

Table 1

Fatty Acid Composition of bPE and Receptor

Fatty acid	% of total fatty acid content bPE	Receptor
C16:0	10.3	16.1
C18:1[a]	7.0	10.4
C18:1[a]	3.6	6.5
C18:0	49.5	47.2
C20:1	2.8	-
C20:4	-	1.0
C18:9OH + C18:10OH	26.8	18.8

[a] Two different C18:1 unsaturated fatty acids

2. Mass Spectrometry

Fast atom bombardment mass spectra (FAB-MS) in negative ion mode were recorded on a VG ZAB-SE magnetic sector instrument. Samples were dissolved in triethanolamine and loaded on the stainless-steel target, which was bombarded with xenon atoms with a kinetic energy of 8 keV and an accelerating voltage of 10 kv was used. Gas-liquid chromatography - mass spectrometry (GLC-MS) in electron ionization mode was carried out on a VG 12-250 quadrupole instrument fitted with a DB-1 capillary column (0.25 mm x 30 m). Spectra were recorded at 70 eV with an ion source temperature of 200°C. For gas-liquid chromatography (GLC), a Hewlett-Packard 5890 instrument was used, equipped with a flame ionization detector. Separations were performed on a DB-1 capillary column (0.25 mm x 30 m).

FAB-MS in negative ion mode of bPE and receptor showed a complicated pattern of $[M-1]^-$ ions in the range of 600-900 mass units (Figure 4). Both compounds showed a considerable heterogeneity in the lipid portion. The molecular weight range is what can be expected for phosphatidylethanolamines. Subtraction of the bPE spectrum from the receptor spectrum indicated that receptor has a different lipid moiety than bPE.

3. NMR Spectroscopy

^1H- and ^{13}C-spectra were recorded on a Bruker AM-500 instrument. Spectra were obtained in CD_3OD at 27°C and chemical shifts were expressed relative to internal tetramethylsilane set to 0 ppm (for ^1H-spectra) or setting the central signal of the methyl-resonance in CD_3OD to 48.9 ppm (for ^{13}C-spectra). Two dimensional proton-proton Correlation Spectroscopy (COSY) and Distortionless Enhancement of Polarisation Transfer (DEPT) spectra were obtained according to Bruker Spectrospin standard software program.

The ^1H-spectra of receptor (Figure 5) showed characteristic signals for a lipid with CH_3 groups (0.85

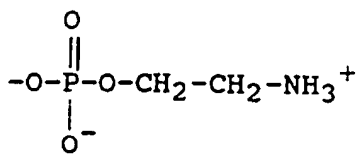
ppm) and CH_2 signals from aliphatic chains (1.3 ppm). Substantial amounts of unsaturations in the fatty acid chains were evident from the bulk of signals around 5.4 ppm. A number of signals not deriving from fatty acids were seen in the area of 6-3 ppm. Two spin systems of equal intensities and with similar features both containing five signals were detected.

The first system with multiplet signal at 5.23 ppm was through cross-peaks in the COSY-spectrum connected to two AB-systems (CH_2-groups judged from their T_1-relaxation), one at 4.43 ppm and 4.17 ppm and the other at 3.95 ppm (signal stemming from two protons). These features bear a close resemblance to glycerol substituted by fatty acids in positions 1 and 2 and a phosphor diester in position 3. (Birdsal et al., J. Chem. Soc. Perkin II:1441-45, 1972; Huang & Andersson, J. Biol. Chem 264:18667-72, 1989). The other spin system showed a similar pattern, CH_2 at 5.17 ppm and a two-proton resonance at 3.98 ppm. The second AB-system showed a marked shift of the other AB-system to 3.0 and 3.95 ppm. This may indicate a change from an acylated to an alkylated CH_2-group.

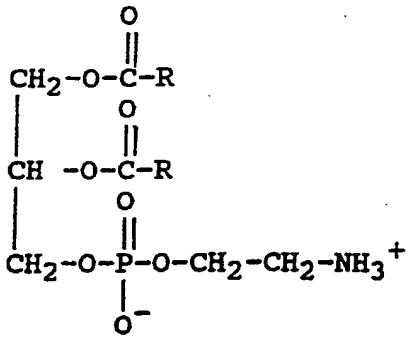
The two remaining signals, a doublet at 5.97 ppm connected to a quartet at 4.35 ppm, indicates a double bond in a structural element of:

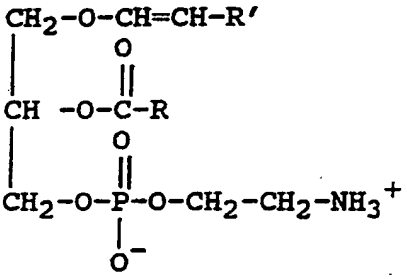
$$-O-CH=CH-CH_2$$

Finally, two multiplets at 4.03 ppm and at 3.15 ppm in the ratio of 4:1 compared to the two glycerol units can be explained by the two CH_2-groups in a phosphorylated ethanolamine:



The above data suggest the following two structures in a 1:1 ratio:





R and R′ represent the hydrocarbon (or hydroxylated hydrocarbon) chains of fatty acids and plasmalogens, respectively. This interpretation was confirmed by recording a reference spectrum of L-α-phosphatidyl-ethanolamine obtained from bovine brain (bPE) and stated to contain 54% plasmalogen (i.e., α, β unsaturated alkyl chains). The fit between the two spectra (receptor and bPE) was almost perfect, confirming the proposed structures. (The reference contained about 67% plasmalogen rather than 54% as stated.) ^{13}C-spectra supported the above structures.

Example 3

Purification of Chlamydial Adhesin

Purified elementary bodies are sonicated 6 times on ice for 30 seconds each at a setting of 4 on a Bronson Sonicator (Model 250 Sonifier, Bronson Ultrasonics Corp., Danbury, Conn.) The sonic extract is radioiodinated according to Example 1.B., and diluted 1:10 in 50 mM Tris-HCl, pH 7.8, containing 150 mM NaCl, and 1.0% bovine serum albumin (TBS-BSA). Other buffers may also be used and,

when it is necessary to prevent the aggregation of proteins, a detergent, such as octyl glucoside, at a concentration of about 0.05% - 0.1% is also included during preparation of the sonic extract.

The chlamydial sonic extract is added to microtiter wells which have been previously coated and fixed with human *Chlamydia* receptor. For example, 0.8-1.0 micrograms of receptor is immobilized in wells as follows. The receptor glycolipids are serially diluted in 25 µl of methanol containing 100 ng each of the auxiliary lipids phosphatidylcholine and cholesterol in flat bottom wells of polyvinylchloride microtiter plates (Falcon 3912-III, Becton-Dickinson). After the solutions are dried by evaporation, the wells are subsequently blocked for 2 hours with Tris-BSA and then rinsed with saline.

After the labeled sonic extract is incubated in receptor-coated wells for 2 hours at room temperature, the wells are washed 5 times with ice-cold saline. To elute ^{125}I-receptor-bound adhesin, the wells are finally incubated 30 minutes at 37°C with 0.05 ml of 10 mM Tris-HCl, pH 7.8, containing 0.1% sodium dodecyl sulfate (SDS). The SDS elution buffer is removed from the wells and the protein is analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography to detect ^{125}I-labeled protein which was bound by receptor and eluted from the plate.

Alternatively, the adhesin is purified by using an affinity chromatography column where the *Chlamydia* receptor is immobilized onto an appropriate gel solid support, such as a hydrophobic gel support, e.g., octyl-agarose (Pharmacia, Piscataway, N.J.). A suitable affinity matrix is prepared by adsorbing the receptor to a hydrophobic gel in the presence of salts (e.g., as described for other lipids by Hirabayashi et al., J. Biochem. 94:327-330, 1983). Photoactivatable hetero-bifunctional crosslinking agents may also be used to prepare lipid affinity matrices (e.g., Lingwood, J. Lipid

Res. 25:1010-1012, 1984). By the use of such agents, the receptor is covalently attached to the gel support.

The sonic extract is loaded on the top of the gel and the column is washed to remove unbound material. The adhesin is then eluted with a chaotropic agent such as NaCl or KSCN, dialyzed and analyzed by SDS-PAGE and autoradiography. An unradiolabeled sonic extract may be processed by any of the above methods subsequent to, or parallel with, the radioiodinated extract in order to provide unlabeled, purified adhesin protein.

Example 4

Binding of Helicobacter Organisms to Lipids

A. Growth of *H. pylori*

H. pylori isolates LC3 and LC11 were cultured from gastric mucosal biopsy samples from children with antral gastritis. The organisms were stored in brucella broth with 10% glycerol and 10% fetal bovine serum at -70°C. Cultures are typically stored for a maximum of 6 months. Cultures were plated on Skirrow's medium and incubated at 37°C under reduced oxygen for 24 h. An inoculum from the plate was placed in 10 ml brucella broth supplemented with 10% fetal bovine calf serum in a disposable Erlenmeyer flask. The flask was placed with a loose screwtop in an evacuation jar and was incubated under reduced oxygen at 37°C for 16 h with constant shaking at 70 rotations per minute. This method consistently resulted in growth of 10^4 organisms per ml. The bacteria had a classic spiral, flagellate morphology when viewed under phase contrast microscopy. They were positive for urease, oxidase, and catalase.

B. Production of Antibodies to *H. pylori*

A whole cell *H. pylori* polyvalent antibody was produced by intravenous injection of a 1500 g New Zealand white rabbit with formalized *H. pylori* strain LC3. Bacteria (10^4) in 0.5 ml phosphate-buffered saline were injected, followed 2 and 6 weeks later by injections of 10^8 organisms suspended in 1 ml phosphate-buffered saline. Antiserum was obtained by way of a venous catheter (with the rabbit under general anaesthesia) two weeks after the last injection. The presence of *H. pylori* antibody was shown by immunoblots of whole cell sonicates of six separate *H. pylori* isolates. The antibody reacted with the same proteins from each isolate.

C. Chromatogram Overlay Assay for Binding of *H. pylori* to Lipids

Lipid extracts (50 µg) were separated by thin-layer chromatography on plastic-backed sheets (Polygram SIL-G, Brinkman Instruments, Ontario, Canada) in chloroform/methanol/water, 65:25:4 (by volume). The plates were blocked in 3% gelatin at 37°C for 2 h. After washing, the plates were incubated at room temperature in a carbon dioxide/hydrogen atmosphere with freshly cultured *H. pylori* in growth medium (10^6/ml). After 2 h incubation, the plates were washed in 100 mmol/l "tris" saline pH 7.6, incubated in a 1/600 dilution of rabbit *H. pylori* antiserum, and incubated for a further 2 h at room temperature. The plates were washed again and incubated with goat antibody to rabbit immunoglobulin conjugated with horseradish peroxidase (Bio-Rad, Richmond, Cal.) for 1 h at room temperature. After washing, bound organisms were visualized by addition to peroxidase substrate 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) Incubations in the absence of *H. pylori* were carried out simultaneously.

Example 5

PURIFICATION AND CHARACTERIZATION OF *H. PYLORI* RECEPTOR

A. Purification

Lipids were extracted from outdated red blood cells, mucosal scrapings of pig stomach and of human stomach obtained at necropsy, and cultured HEp2 cells. The tissue was weighed, homogenized in a minimum volume of water, and extracted in 20 volumes of chloroform/methanol 2:1 (by volume); the extract was then partitioned against water. The lower-phase lipids were dried, dissolved in chloroform/methanol 98:2, and applied to a silicic acid column previously equilibrated in chloroform. The column was washed extensively in sequence with chloroform, acetone/methanol 9:1 (3:1 for red blood cell extracts), and methanol. The fractions were dried and weighed. For further purification, the methanol fraction, which contained the *Helicobacter*-binding lipid, was concentrated, reapplied to a silicic acid column, and eluted with a linear polarity gradient of chloroform/methanol 10:1 to 2:1. Fractions were concentrated and tested for binding by the thin-layer chromatography overlay assay as described in Example 4. Those containing the receptor were pooled.

B. Analyses

Phospholipids of human red blood cells and *H. pylori* receptor were separated by HPLC (as described by Heinz et al., Chromatographia 25:497-503, 1988). Fatty acids of the receptor phospholipids were analyzed by phospholipase C digestion methanolysis, HPLC, and GLC separation and peak integration as described by Myher et al. (Lipids 24:396-407, 1989). The results of fatty acid analysis of phosphatidylethanolamine from red blood cells ("RBC PE") and receptor are summarized in Table 2.

Table 2

Fatty Acid Composition of RBC PE and Receptor

FAME + DMA FAME[1]	RECEPTOR AREA %	RBC PE AREA %
"16:0"	15.05	12.18
"17:0"	0.32	0.21
"18:0"	8.71	6.11
"18:1"	19.69	15.79
"18:2w6"	4.44	5.37
"20:1w9"	0.61	0.30
"20:2w6"	0.40	0.21
"20:3w6"	1.01	0.85
"20:4w6"	17.67	20.34
"20:5w3"	0.10	0.60
"22:4w6"	5.15	5.58
"22:5W3"	0.59	0.87
"22:5w3"	2.12	3.19
"22:6w3"	3.33	4.64
DMA[2]		
"16:0"	5.05	6.33
"17:0"	0.50	0.91
"18:0"	11.01	11.85
"18:1"	4.24	4.66

[1] Fatty acid methyl esters

[2] Dimethylacetals

Based on the results of the structural characterization of *H. pylori* receptor, it is a phosphatidylethanolamine-like molecule. When native phosphatidylethanolamines are tested in the chromatogram overlay assay (described in Example 4.C. above), considerable variation in the binding of *H. pylori* to phosphatidylethanolamine (PE) from different sources was observed. Of the native PEs, the PE from *E. coli* was the most effective receptor. PE from bovine brain, porcine liver, egg yolk, and soy bean were recognized to a lesser degree by *H. pylori*, while PE from bovine liver and dog brain showed no receptor activity.

Example 5

CLONING OF ADHESIN DNA

A. Production of Monoclonal Antibodies That Inhibit Adhesion

Balb/C mice are immunized with membranes from a microorganism possessing an adhesin, and their sera are tested for the development of antibody that inhibits the membranes from binding to receptor. Spleens from these mice are used to isolate splenocytes for fusion with SP2/O-AG14 (ATCC CRL 8287) mouse myeloma cells (according to Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Positive fusion hybridoma cultures from separate fusions are screened for the production of antibody that reacted on ELISA with the membranes. The ELISA is performed as follows: Membranes containing 1 μg of protein are used to coat 96-well microtiter plates. The coated wells are washed with PBS (phosphate buffered saline, 10 mM sodium phosphate, pH 7.5, 167 mM sodium chloride), then incubated with serial 100 μl of hybridoma culture supernatant. The walls are washed, incubated with 100 μl of secondary goat anti-mouse antibody conjugated with horseradish peroxidase for 1 h, then bound antibody is detected colorimetrically (Biorad, Richmond, Calif.). Membrane-reactive hybridoma cultures are then tested for the ability to inhibit membrane binding. Hybridoma culture supernatants are incubated with 4 x 10^6 cpm of ^{35}S-methionine labeled membranes for 1 h at room temperature. This mixture is then added to serial dilutions of receptor bound passively to 96-well microtiter plates and assayed for binding. The hybridoma cultures which produce suitable antibodies are cloned by limiting dilution to obtain stable cell lines according to Harlow et al. Large amounts of antibody were produced in the ascites fluid of Balb/C mice, and the class of each antibody was determined according to Harlow et al.

B. Cloning and Sequencing of a Gene That Encodes an Adhesin Protein

Cloning methods are generally performed by standard procedures as described by Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (1982). Total DNA is isolated and partially digested with the restriction enzymes Eco R1 according to the manufacturer's recommendations (Boerhinger-Manheim). DNA fragments 4-15 kbp in length are isolated on a sucrose gradient and ligated to Eco R1-digested Lambda ZAPII arms as supplied by Stratagene, Inc. (La Jolla, Calif.). This ligation is then packaged into phage particles and used to tranfect the *Escherichia coli* host strain, XL-1 (according to Stratagene protocol) to obtain phage plaques which express proteins encoded by the foreign DNA. These plaques are used in an immunoblot screen with anti-adhesin antibody using a Stratagene Picoblue detection kit. Positive reacting plaques are purified and used to induce the production of a plasmid through the use of the helper phage R408 (according to Stratagene protocol). These plasmids carry the insert DNA which encodes the adhesin protein. The location of the gene encoding the protein is determined by deletion analysis. Deletion analysis is performed by generation of subclones containing various restriction fragments in the vector pSK(-) (Stratagene). The adhesin gene in clones testing positive for expression of adhesion protein is DNA sequenced according to the method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977).

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

1. An isolated DNA molecule encoding an adhesin protein.

2. A DNA molecule according to claim 1 wherein the DNA is cDNA or genomic DNA.

3. An isolated DNA molecule capable of specifically hybridizing with a DNA molecule encoding an adhesin protein.

4. A recombinant plasmid comprising a DNA molecule encoding an adhesin protein.

5. The recombinant plasmid of claim 4 wherein the DNA molecule comprises cDNA or genomic DNA.

6. A recombinant plasmid capable of directing the expression of an adhesin protein in a host cell, said plasmid comprising a promoter followed downstream by a DNA molecule encoding an adhesin protein.

7. Cells stably transfected with a recombinant plasmid comprising a DNA molecule encoding an adhesin protein, said cells producing said adhesin protein in recoverable amounts.

8. The stably transfected cells of claim 7, wherein said cells are prokaryotic cells or eukaryotic cells.

9. A method for producing an adhesin protein, comprising:

introducing into a host cell a recombinant plasmid capable of directing the expression of an adhesin protein in

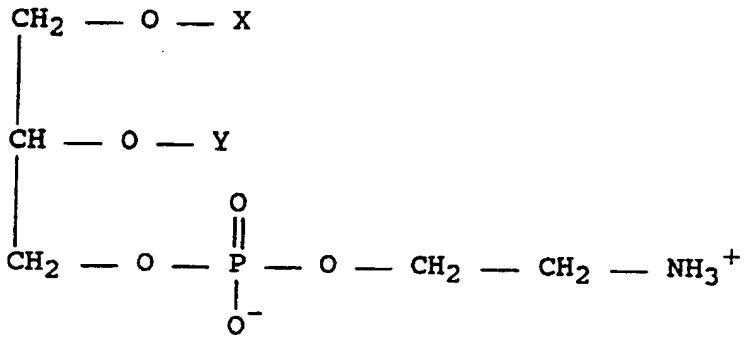
the host cell, said plasmid comprising a promoter followed downstream by a DNA molecule encoding an adhesin protein;

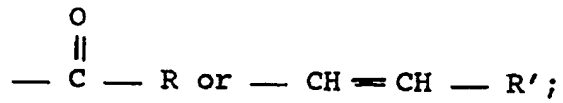
growing said host cell in an appropriate medium; and

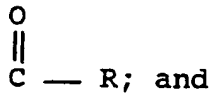
isolating the protein product encoded by said DNA molecule, produced by said host cell.

10. The method of claim 7 wherein the host cell is a prokaryotic cell or a eukaryotic cell.

11. An isolated receptor for a microorganism consisting essentially of a plurality of phospholipids having the formula:



wherein X is  ;

Y is  ; and

R′ is an alkyl group and R are alkyl, hydroxyalkyl or alkenyl groups of fatty acids.

12. A pharmaceutical composition comprising a phospholipid according to claim 11 in combination with a pharmaceutically acceptable carrier or diluent.

13. An isolated receptor for a microorganism characterized by having been prepared by the process consisting essentially of:

extracting the lipids of HeLa cells to yield a lipid extract;

contacting said lipid extract with DEAE-agarose exchange resin under conditions sufficient to permit binding;

washing with methanol said exchange resin to which said lipid extract has been contacted;

eluting a fraction with methanol containing 10-20 mM NH_4HCO_3;

separating said fraction by silica gel preparative thin-layer chromatography;

isolating a band positive for primuline and which binds a microorganism;

washing said band with chloroform; and

eluting said receptor from said band with methanol.

14. A method for screening for a microorganism which lacks an adhesin protein, comprising:

contacting a receptor according to claim 11 with an aliquot of a selected microorganism under conditions and for a time sufficient to allow binding to occur; and

detecting the presence or absence of a bound microorganism, thereby determining the presence or absence of an adhesin protein in said microorganism.

15. The method of claim 14 wherein a reporter group is attached to the microorganism, and wherein the step of detecting comprises removing substantially any unbound microorganism and thereafter detecting the presence or absence of the reporter group.

16. A microbial adhesin protein characterized by having been prepared by the process consisting essentially of:

contacting a receptor according to claim 11 with a microorganism preparation, wherein said preparation contains an adhesin protein, under conditions and for a time sufficient to allow binding between said receptor and said adhesin protein; and

isolating said adhesin protein.

17. The protein of claim 16 wherein the receptor is bound to a solid support, and wherein the step of isolating comprises washing the solid support to remove unbound material and eluting the adhesin protein from the solid support.

18. A purified microbial adhesin protein.

19. A vaccine comprising a protein according to claim 18 in combination with a pharmaceutically acceptable carrier or diluent.

20. An antibody that specifically binds to a protein according to claim 18.

FIGURE 1

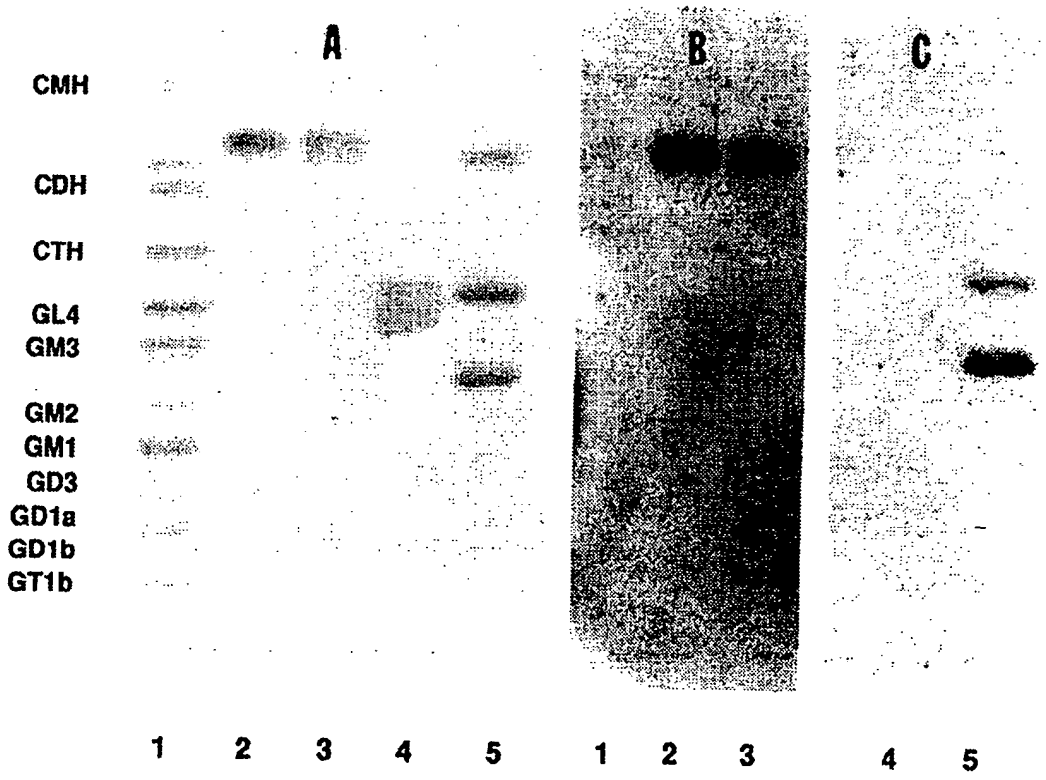


FIGURE 2

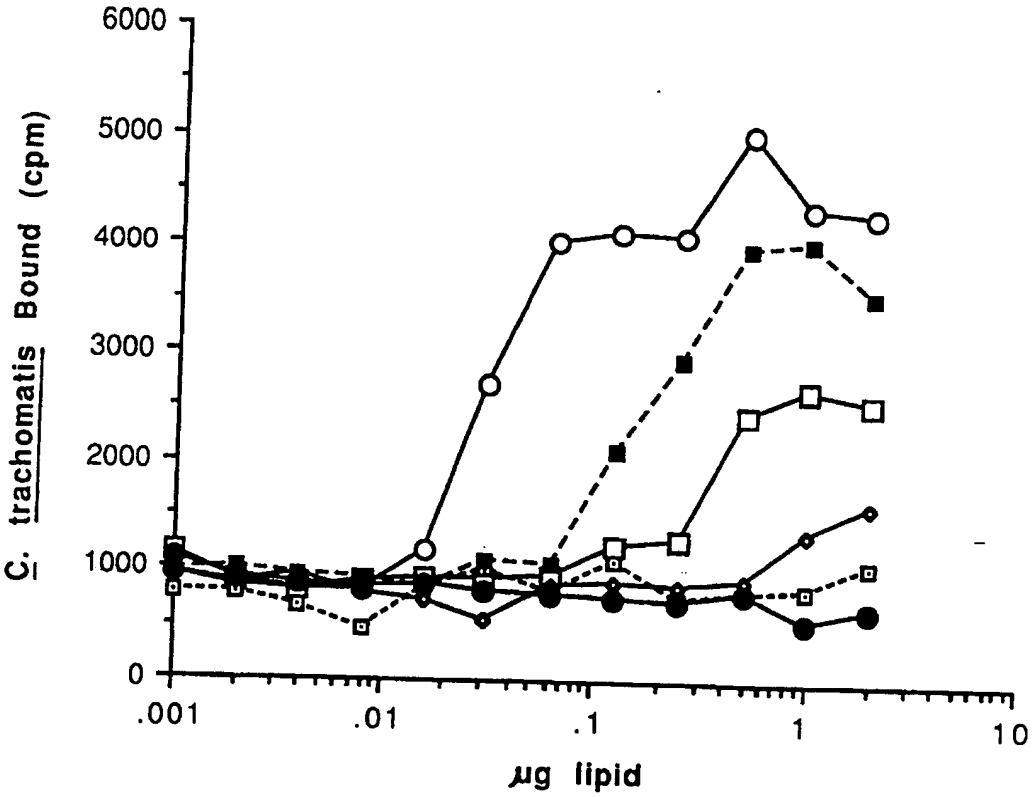

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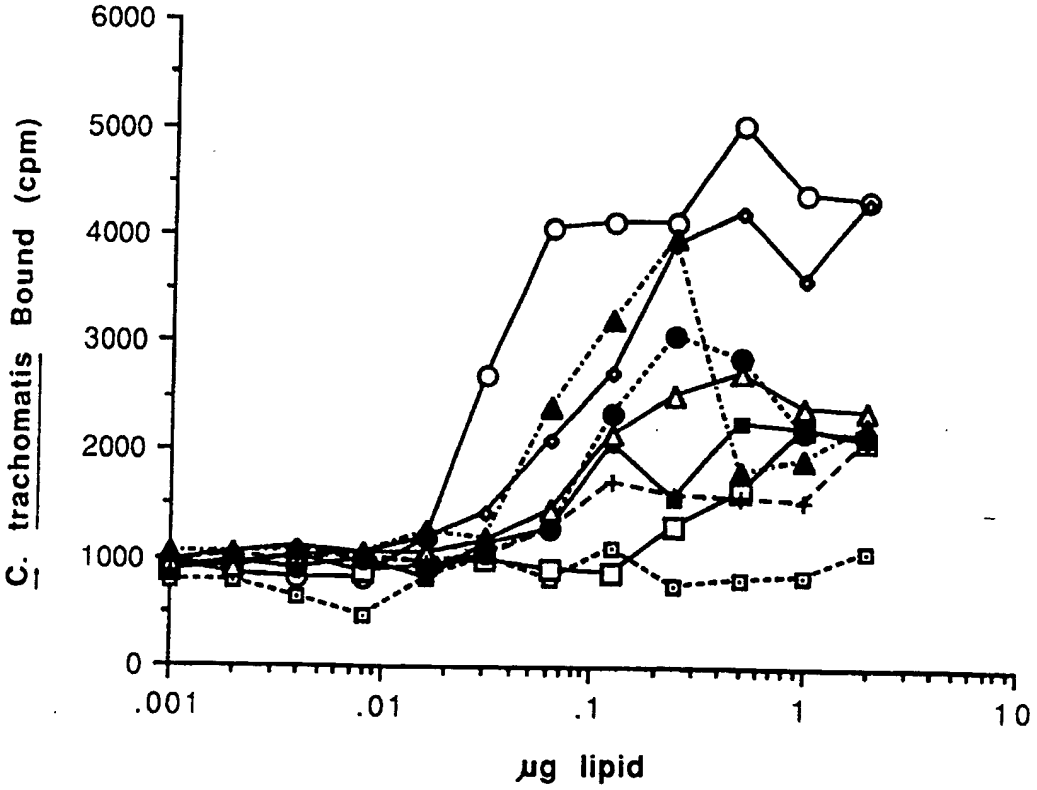
FIGURE 3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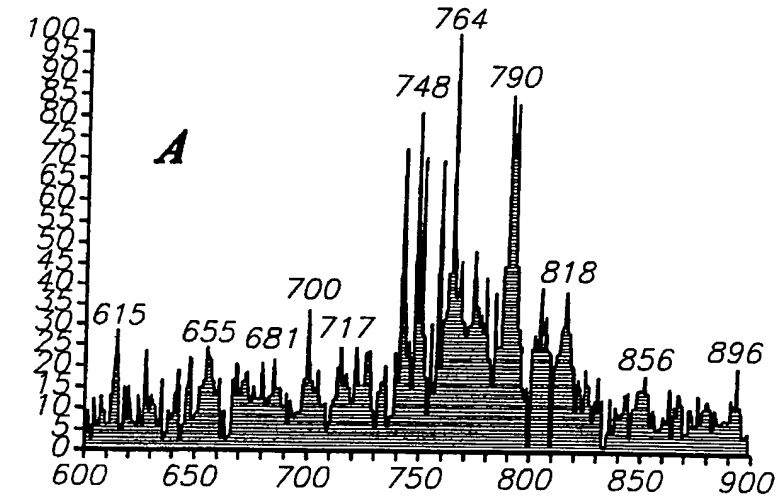
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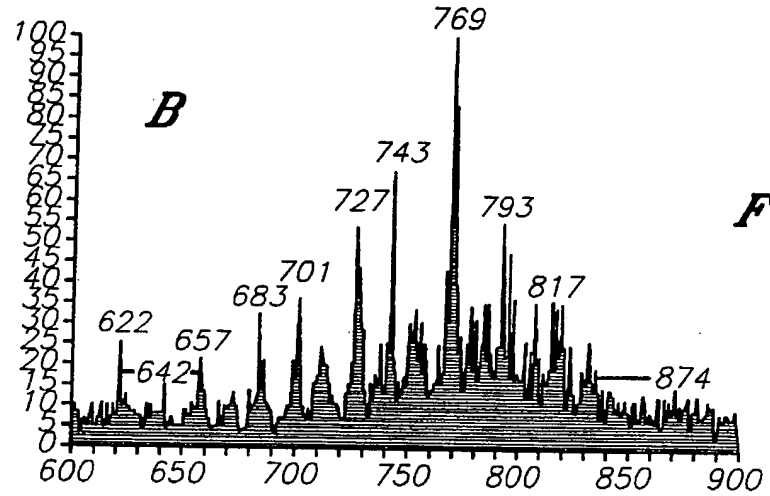
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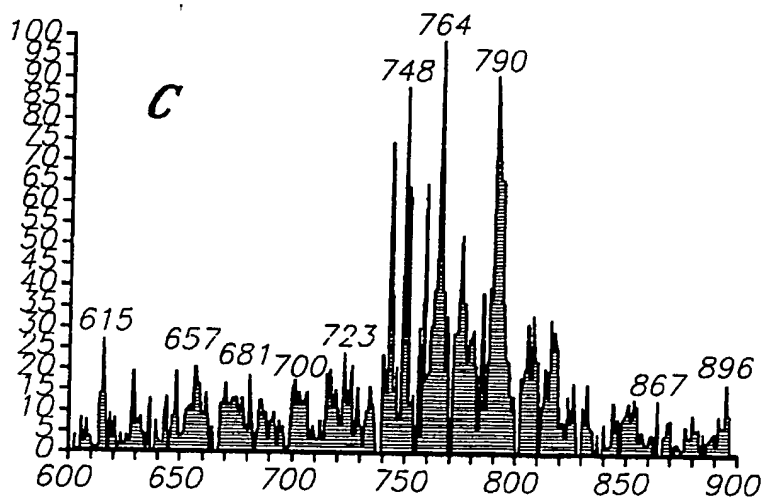
Fig. 4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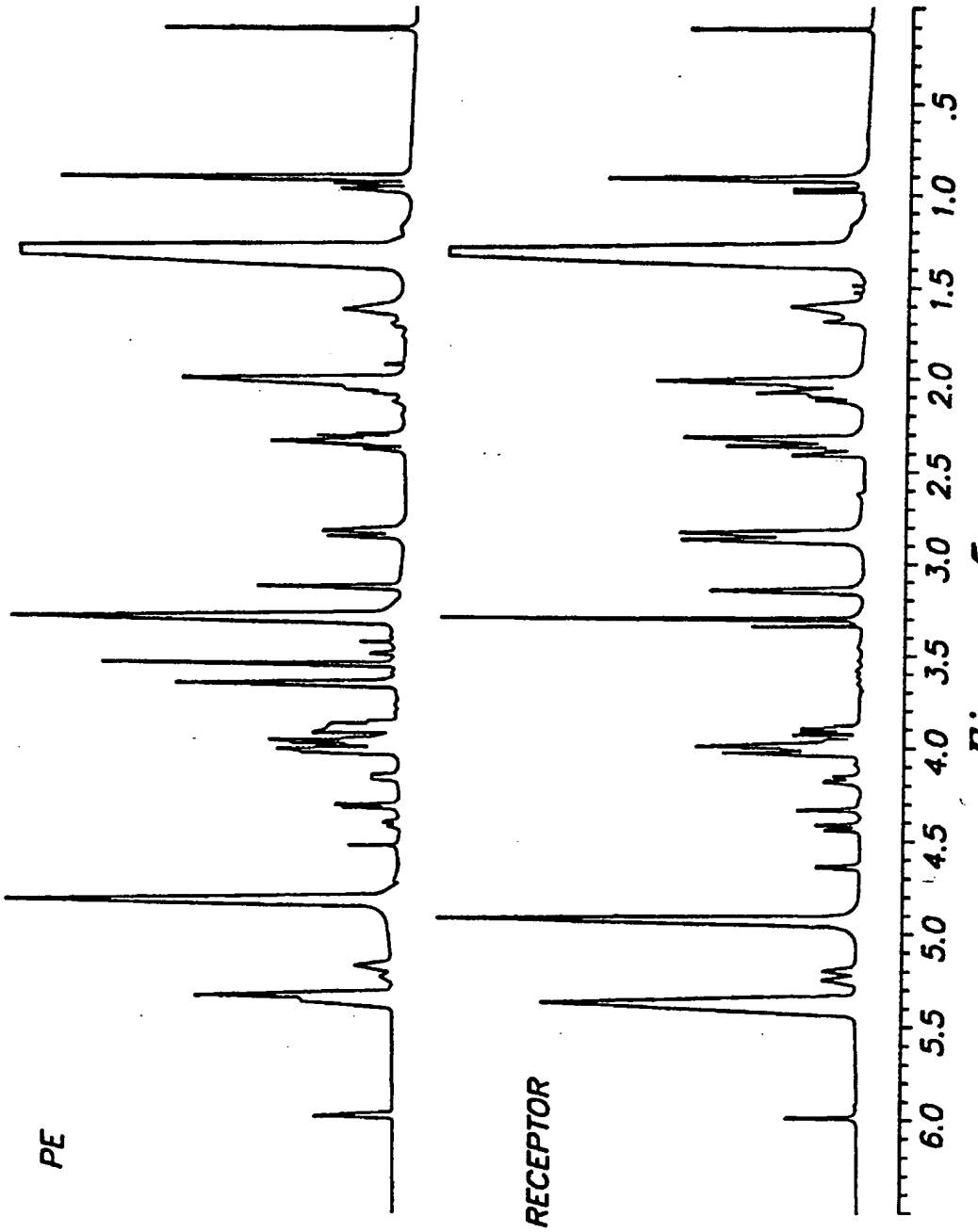

Figure 5